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Title: Chlorophyll *a* is a favorable substrate for *Chlamydomonas* Mg-dechelatase encoded by *STAY-GREEN*

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Abstract

Mg removal from chlorophyll by Mg-dechelatase is the first step of chlorophyll degradation. Recent studies showed that in *Arabidopsis, Stay Green* (*SGR*) encodes Mg-dechelatase. Though the *Escherichia coli* expression system is advantageous for investigating the properties of Mg-dechelatase, *Arabidopsis* Mg-dechelatase is not successfully expressed in *E. coli*. *Chlamydomonas reinhardtii* SGR (CrSGR) has a long, hydrophilic tail, suggesting that active CrSGR can be expressed in *E. coli*. After the incubation of chlorophyll *a* with CrSGR expressed in *E. coli*, pheophytin *a* accumulated, indicating that active CrSGR was expressed in *E. coli*. Substrate specificity of CrSGR against chlorophyll *b* and an intermediate molecule of the chlorophyll *b* degradation pathway was examined. CrSGR exhibited no activity against chlorophyll *b* and low activity against 7-hydroxymethyl chlorophyll *a*, consistent with the fact that chlorophyll *b* is degraded only after conversion to chlorophyll *a*. CrSGR exhibited low activity against divinyl chlorophyll *a* and chlorophyll *a*′ , and no activity against chlorophyllide *a*, protochlorophyll *a*, chlorophyll c_2, and Zn-chlorophyll *a*. These observations indicate that chlorophyll *a* is the most favorable substrate for CrSGR. When CrSGR was expressed in *Arabidopsis* cells, the chlorophyll content decreased, further confirming that SGR has Mg-dechelating activity in chloroplasts.

1. Introduction

Land plants and green algae have antenna complexes that absorb light energy for photosynthesis. The level of these complexes changes in response to light conditions (Schöttler and Toth, 2014). When plants are subjected from low to high light conditions, the antenna complex is degraded to decrease the absorbance of light energy and prevent photodamage. The developmental stage of a plant also affects the level of this chlorophyll protein complex. In senescent leaves, the chlorophyll protein complex, which is one of major nitrogen source, is degraded, and its nitrogen is exported to the growing organs (Hörtensteiner and Feller, 2002). In this process, chlorophyll degradation is thought to trigger chlorophyll protein degradation, because mutants defective in chlorophyll degradation do not degrade chlorophyll proteins (Bachmann et al., 1994; Wu et al., 2016). Understanding the enzymes and the regulation of chlorophyll degradation is therefore essential for gaining insight into the process of chlorophyll protein degradation.

The extraction of a central Mg is the first step of chlorophyll degradation (Hortensteiner, 2006) (Fig. 1), and this reaction is the committed step of chlorophyll degradation. The extraction of Mg from chlorophyll is catalyzed by Mg-dechelatase, and recent studies show that it is encoded by *Stay Green* (*SGR*) (Shimoda et al., 2016). *SGR* was initially identified in stay-green mutant plants (Park et al., 2007; Christ and Hörtensteiner, 2014). In many of the plants examined, the *SGR* mutants preserve chlorophyll during senescence. This is consistent with the fact that SGR is the Mg-dechelatase that catalyzes the first step of chlorophyll degradation. *SGR* homologs are found in green algae and land plants. In land plants, *SGR* homologs can be classified into two subfamilies, the *SGR* subfamily, and *SGR-like* (*SGRL*) subfamily. *Arabidopsis* has two SGR, *Arabidopsis* SGR1 (*AtSGR1*) and *Arabidopsis* SGR2 (*AtSGR2*) and one *Arabidopsis* SGRL (*AtSGRL*). These three *Arabidopsis* SGR homologs remove Mg from chlorophyll *a*, but

none of them removes Mg from chlorophyll *b* (Shimoda et al., 2016). This substrate specificity serves to degrade chlorophyll *b* correctly. For degradation, chlorophyll *b* converts to chlorophyll *a* (Hortensteiner, 2006). Therefore, chlorophyll *a* is believed to be the only chlorophyll from which Mg is removed in the chlorophyll degradation process. AtSGR1 and AtSGR2 do not remove Mg from chlorophyllide *a*, whereas, AtSGRL does (Shimoda et al., 2016). The molecular mechanism that causes this difference in substrate specificity is not yet understood.

Mg-dechelatase is an important enzyme for understanding the regulation of chlorophyll degradation, although its enzymatic character remains unclear. *Arabidopsis* SGR was investigated using the recombinant protein expressed by the wheat germ cell-free protein expression system (Shimoda et al., 2016). This system is not as useful as the conventional *Escherichia coli* expression system, because its protein yield is much lower. Low protein yields cause difficulties when examining the enzymatic properties under various conditions and against various substrates with the consistency lot of the recombinant protein. Among the photosynthetic eukaryotes, *Arabidopsis* and *Chlamydomonas* are model organisms. *Chlamydomonas reinhardtii* SGR (CrSGR) has a long hydrophilic tail. The presence of this long tail may allow it to form an active enzyme in *E. coli.* In this study, expression of the active CrSGR in *E. coli* was succeeded and the enzymatic properties of CrSGR were investigated.

2. Materials and methods

2.1. CrSGR preparation

Chlamydomonas reinhardtii (*arg7*/*cw15*) was obtained from Chlamydomonas Genetic Center. CrSGR (Cre12.g487500) lacking its transit peptide was amplified by PCR using the primers sets (CrSGR; forward 5′ -AAGGAGATATACATATGGCCTCCAGGCGACAACCT-3′ , CrSGR reverse1; 5′ -TCGTCATCGTCTTTGTAGTCGGAGGCCGAGGAGCGCGCTA-3′). The FLAG-tag DNA sequence was added to the 3′ end of CrSGR by PCR using the primes sets (CrSGR; forward 5′ -AAGGAGATATACATATGGCCTCCAGGCGACAACCT-3′ , CrSGR reverse2; 5′ -GGTGGTGGTGCTCGACCTTGTCGTCATCGTCTTT-3′). cDNA was cloned into pET-30a (+) (Novagen) using the *Nde*I and *Xho*I sites with an In-Fusion cloning system (Clontech). The expression plasmid was introduced into *E. coli* Rosetta (DE3). CrSGR was expressed at 37 °C for 3 h with 0.4 mM isopropyl-β-*o*-thiogalactopyranoside. Two hundred mL of the culture was harvested by centrifugation at 5,000 ×*g* for 5 min and then resuspended in 10 mL of BugBuster Protein Extraction Reagents (Novagen) containing 10 µL of benzonase (Novagen). The culture lysate or the soluble fraction of the culture lysate, obtained by centrifugation at 22,000 ×*g* for 5 min, were mixed with the same volume of the sample buffer (25 mM Tris-HCl, pH 6.8, 4% [w/v] SDS, 10% [w/v] sucrose, 5% [v/v] 2-mercaptoethanol, and trace amounts of bromophenol blue) and were denatured by heating at 95 °C for 3 min. The culture lysate and the soluble fraction were subjected to SDS-PAGE. After electrophoresis, proteins were stained with Coomassie Brilliant Blue (CBB), or transferred to the polyvinylidene difluoride membrane for immunoblotting analysis. Antibodies against FLAG-tag (Sigma-Aldrich) detected FLAG-tagged CrSGR.

2.2. Preparation of chlorophyll derivatives

Pheophytin derivatives were prepared by mixing pigments with 0.1% (v/v) of 1N HCl in acetone. Divinyl chlorophyll *a* was extracted from an slr1923-deficient *Synechocystis* mutant (Ito et al., 2008). 7-Hydroxymethyl chlorophyll *a* was obtained by the reduction of chlorophyll *b* with 1 mM $NaBH_4$ in methanol (Holt, 1959). Chlorophyllide *a* was prepared from chlorophyll *a* through hydrolysis with recombinant chlorophyllase (Tsuchiya et al., 1999). Protochlorophyll *a* was synthesized by the chemical oxidation of chlorophyll *a* with 0.5 mM 2,3-dichloro-5,6-dicyanobenzoquinone in diethyl ether (Shedbalkar et al., 1991). Zn-chlorophyll *a* was prepared from pheophytin *a* solubilized in dichloromethane, by mixing 1/10 volume of zinc acetate saturated methanol (Kunieda and Tamiaki, 2009). Chlorophyll *a*′ was prepared from chlorophyll *a* in triethylamine (Watanabe et al., 1987). Chlorophyll c_2 was prepared from dinoflagellates *Amphidiniella sedentaria* (Horiguchi, 1995) (a kind gift from Prof. T. Horiguchi, Hokkaido University). Chlorophyll derivatives used for the enzymatic analysis were purified, using thin-layer chromatography plates silica gel 60 (MERCK) developed with petroleum ether: acetone (7:3 v/v). The molar extinction coefficient of divinyl chlorophyll in 80% acetone at 664 nm is 69.29 (Shedbalkar and Rebeiz, 1992), 7-hydroxymethyl chlorophyll *a* in diethyl ether at 655.5 nm is 61.1 (Ito et al., 1996), protochlorophyll *a* in 80% acetone at 432 nm is 241 (Kahn, 1983), chlorophyll c_2 in 90% acetone with 1% pyridine at 443.8 nm is 227 (Jeffrey, 1972), Zn-chlorophyll *a* in diethyl ether at 653 nm is 90 (Jones et al., 1968).

2.3. Enzyme assay

A culture lysate expressing CrSGR (10 µL) was suspended in 40 µL of a reaction buffer (20 mM sodium phosphate, pH 7.5, 100 mM NaCl, 0.1% Triton X-100). The pigments were solubilized in acetone, and less than 1.5 µL of the acetone solution was added to the reaction buffer. The final concentration of the pigments used for the reaction was 2.5 to 10.0 µM. The reaction mixtures were incubated for 30 min at 25 °C and reactions were stopped by the addition of 200 µL of acetone. The pigments were analyzed by HPLC using a C8 column (Waters Symmetry C8, Waters) with a gradient from eluent A (methanol:acetonitrile:aquaeous pyridine solution [0.25 M pyridine] [50:25:25 v:v:v]) to eluent B (methanol:acetonitrile:acetone [20:60:20 v:v:v]) at a flow rate of 1 mL min^{-1} at 40°C (Shimoda et al., 2012). When the optimal pH and the kinetics parameters were determined, the column was developed with eluent B. The elution profiles were monitored by measuring their fluorescence (RF-20A, Shimadzu). Pigment quantification was performed using the areas of the peaks. Chlorophyll *a*′ was analyzed on a normal-phase HPLC system. The column (Senshupak Silica 2141-N, Senshu Science) was developed with a solvent (hexane: toluene: methanol =100:4:0.8 [v/v]), at a flow rate of 1 mL min^{-1} at 25 °C (Nakamura et al., 2001).

2.4. CrSGR expression in *Arabidopsis*

The transit peptide of *Arabidopsis SGR1* (AT4G22920) and *CrSGR* without its transit peptide were amplified by PCR using primer sets (*Arabidopsis* transit forward; 5′ -AAAGCAGGCTCCACCATGTGTAGTTTGTCGGCGAT-3′, *Arabidopsis* transit reverse; 5′ -GCCTGGAGGCAACGGGAACAATCGATTGGT-3′) and (CrSGR forward; 5′ -TGTTCCCGTTGCCTCCAGGCGACAACCTAC-3′, CrSGR reverse; 5′

-AAGCTGGGTCTAGATTCACTTGTCGTCATCGTCTTTGT-3′). Amplified fragments were mixed and further amplified using the primer sets of *Arabidopsis* transit forward and CrSGR reverse. The amplified fragments of CrSGR with *Arabidopsis* SGR1 transit peptide were introduced into the Gateway entry vector pENTR4 Dual (Invitrogen), between the *Nco*I and *Eco*RV sites, and then introduced into the Gateway-compatible inducible vector pOpOn (Shimoda et al., 2016), which was constructed from pOpOff2 (Craft et al., 2005; Wielopolska et al., 2005) by removing the antisense fragments. Transgene expression was driven by the pOp6 promoter. The construct was introduced into *Agrobacterium tumefaciens* (strain GV3101) and transformed into *Arabidopsis thaliana* (Columbia ecotype). Transgenic plants were grown at 25 °C, under continuous light conditions, at a light intensity of 80 µmol photons m^{-2} s^{-1}. Excised leaves of three-week-old plants were sprayed with 10 µM dexamethasone (DEX) supplemented with 0.05% (v/v) Silwet L-77 and put on wet paper. After 24 h, leaves were homogenized in 20 volumes (v/w) of the sample buffer described above for protein extraction, or homogenized in 40 volumes (v/w) of acetone for chlorophyll extraction. Proteins were subjected to SDS-PAGE and immunoblotting analysis. Chlorophyll content was determined by spectrophotometry (U-3310, Hitachi) (Porra et al., 1989).

2.5. Sequence analysis

The database search was performed using Phytozome 11, JOINT GENOME INSTITUTE. Amino acid sequences were aligned using the ClustalW program in the BioEdit Program (Hall, 1999). The phylogenic tree was constructed using MEGA5 (Tamura et al., 2011).

3. Results

3.1. Expression of CrSGR in *E. coli*

SGR homologs of two green algae, *Chlamydomonas*, *Micromonas*, and four land plants, *Arabidopsis*, *Solanum*, *Oryza,* and *Sorghum*, were aligned and a phylogenic tree was constructed (Fig. 2). Conserved amino acids in land plants are found in green algae. Both the SGR subfamily and SGRL subfamily are found in monocot and dicot plants. The *Chlamydomonas* SGR homolog and *Micromonas* SGR homolog were phylogenetically divergent from the land plants and were not included in the SGR or SGRL subfamily. Green algae SGR homologs extended their C-terminal (Fig. 2A). CrSGR had a notably long tail. Active *Arabidopsis* SGR was not expressed in *E. coli*, however, it was expected that the structurally different CrSGR would be expressed in *E. coli*. Thus, CrSGR was examined to determine if the active CrSGR protein could be expressed in *E. coli*. After the induction of CrSGR in *E. coli*, proteins were subjected to gel electrophoresis. In the CBB stained gel, the band corresponding to the CrSGR protein, of which the predicted molecular size is 62.4 kDa, was not identified (Fig. 3). Proteins were also subjected to immunoblotting analysis. CrSGR was detected in the cell lysate and the soluble fraction (Fig. 3). Although the levels were low, CrSGR was expressed in *E. coli*. Next, Mg-dechelatase activity was examined using the cell lysate and the soluble fraction. Pheophytin *a* accumulated after incubation of chlorophyll *a* with the cell lysate or the soluble fraction for 30 min (Fig. 4). These observations demonstrated that CrSGR is Mg-dechelatase and can be expressed in *E. coli*. The cell lysate exhibited higher Mg-dechelatase activity

than the soluble fraction. Therefore, the cell lysate was used in the following experiments.

3.2. Determination of the optimal pH and the kinetic parameters

Mg is removed under weak acidic conditions (Saga and Tamiaki, 2012). Therefore, if CrSGR directly uses protons derived from the solution to remove Mg, it is active under these weak acidic conditions, whereas, if the active site of CrSGR is not exposed to the solution and protons are derived from the acidic amino acid residues, it is active under neutral conditions. The optimal pH of CrSGR was investigated over a pH range of 6.0 to 9.5 using 6 μM of chlorophyll *a* as a substrate (Fig. 5A). *E. coli* lysate was diluted four fold in the corresponding pH buffer. The optimal pH of CrSGR was 7.5, suggesting that the acidic amino acid residues are proton donors. To determine the K_m value for chlorophyll *a*, pheophytin *a* production was measured over a range of chlorophyll *a* concentrations at the optimal pH (Fig. 5B). The K_m for chlorophyll *a* was calculated as 8.44 μM and V_{max} was 0.16 μM min^{-1}.

3.3. Substrate specificity of CrSGR

The study of substrate specificity advances our understanding of the biochemical properties and physiological functions of CrSGR. Natural and artificial chlorophyll derivatives (Fig. 6) were examined to determine if they were substrates of CrSGR (Fig. 7).Chlorophyll *a* was incubated with the lysate of *E. coli* possessing an empty vector. A small peak, eluted with an identical retention time as pheophytin *a,* was detected before incubation. Chlorophyll *a* used for this experiment contained a small amount of pheophytin *a*. After incubation for 30 min, pheophytin *a* did not increase, suggesting that chlorophyll *a* was stable during incubation, and the *E. coli* cell lysate did not remove Mg from chlorophyll *a*. When chlorophyll *a* was incubated with the lysate of the cell expressing CrSGR, pheophytin *a* increased; however, when chlorophyll *b* was incubated with the cell lysate, pheophytin *b* was not detected. This observation is consistent with findings of previous reports where AtSGR did not remove Mg from chlorophyll *b*. Chlorophyllide *a* is the precursor of chlorophyll *a* in the chlorophyll biosynthetic pathway. When chlorophyllide *a* was incubated with the cell lysate, pheophorbide *a* was not detected, showing that chlorophyllide *a* was not a substrate of CrSGR. This result is similar to the activities of AtSGR1 and AtSGR2. 7-Hydroxymethyl chlorophyll *a* has a hydroxymethyl group at the C-7 position. After the incubation of 7-hydroxymethyl chlorophyll *a* with the cell lysate, the product (7-hydroxymethyl pheophytin *a*) increased. The ethyl group at the C-8 position is substituted with the vinyl group in divinyl chlorophyll *a*. After the incubation of divinyl chlorophyll *a* with the cell lysate, the product (divinyl pheophytin *a*) increased. Both 7-hydroxymethyl chlorophyll *a* and divinyl chlorophyll *a* could be substrates of CrSGR, but Mg was removed from them less efficiently than from chlorophyll *a*. Chlorophyll *a*′, the C-13^2 epimer of chlorophyll *a*, was found in the photosystem I reaction center. When the chlorophyll *a*/*a*′ mixture was incubated with the cell lysate, both of them were converted to corresponding pheophytin derivatives. Compared to chlorophyll *a*, chlorophyll *a*′ was less efficiently converted to pheophytin *a*′, suggesting that the C-13^2 side chain affects the substrate recognition, or activity of CrSGR. Protochlorophyll *a* is an artificial chlorophyll and its D-ring is oxidized. After incubation of protochlorophyll *a* with the cell lysate, the pheophytin derivative (protopheophytin *a*) was not detected. Chlorophyll c_2 is the accessory pigment of some diatoms, brown algae, and dinoflagellates. After incubation of chlorophyll c_2 with the cell lysate, the

pheophytin derivative (pheophytin c_2) was not detected. Zn-chlorophyll *a* possesses Zn as the central metal ion instead of Mg. After incubation of Zn-chlorophyll *a* with the cell lysate, pheophytin *a* was not detected. This may be because Zn coordinates to the chlorin ring stronger than Mg. CrSGR may not be able to remove Zn from this stronger coordinate bond. The substrate specificity is summarized in Table 1. These observations demonstrated that chlorophyll *a* is the most favorable substrate for CrSGR.

3.4. CrSGR expression in *Arabidopsis*

CrSGR exhibited the same substrate specificity with AtSGR1 and AtSGR2, against chlorophyll *a*, chlorophyll *b* and chlorophyllide *a*. This indicates that their functions are conserved between *Chlamydomonas* and *Arabidopsis*. To examine if CrSGR is active in land plants, CrSGR was transiently expressed in *Arabidopsis* using a chemically induced system (Fig. 8). CrSGR accumulation was determined with immunoblotting analysis. After induction for 24 h, chlorophyll was degraded in *Arabidopsis* leaves, demonstrating CrSGR activity as Mg-dechelatase in *Arabidopsis* cells.

4. Discussion

4.1. Availability of *E. coli* expression system for SGR preparation

Stay-green mutants are attracting widespread interest in agriculture. The genetic and physiological routes by which they act are diverse (Thomas and Howarth, 2000). One of the responsible genes for stay green was suggested in 2006 by genetically mapping forage grasses (Armstead et al., 2006) and identified in 2007 by using mutant rice (Park et al., 2007; Christ and Hörtensteiner, 2014). The *SGR* gene was also identified as the encoded gene on the *I* locus in Mendel's law (Sato et al., 2007). Mg-dechelatase was the unidentified gene in the chlorophyll degradation pathway when *SGR* was identified. However, it took about 10 years to demonstrate that SGR is actually Mg-dechelatase (Shimoda et al., 2016). The reason for this delay in the identification and elucidation of the function of SGR is two-fold. First, cell free protein expression systems are necessary for the preparation of active AtSGR and conventional *E. coli* expression systems had not yet been successful in preparing active SGR. Second, the actual substrate of AtSGR1 has not been used to examine the enzymatic activity of the recombinant SGR. Chlorophyllide or chlorophyllin are often used instead of chlorophyll as the substrates for enzymatic assays (Suzuki et al., 2005), because they are more soluble in the aqueous solution and using them is often successful. For example, although chlorophyll *b* is believed to be the substrate of chlorophyll *b* reductase in vivo, chlorophyllide *b* is the better substrate in vitro (Shimoda et al., 2012). In this study, CrSGR was successfully expressed in *E. coli*. The molecular size of CrSGR, without a transit peptide, is 61.3 kDa. This is larger than that of AtSGR1 without the transit peptide (24.7 kDa). The C terminal of CrSGR was extended (Fig. 2). This long tail is thought to affect the expression of the active enzyme in *E. coli*. The expression of SGR in *E. coli* allows us to characterize it in detail. In order to purify CrSGR, a histidine tag was introduced into the C terminal of CrSGR. However, CrSGR could not be purified using a nickel column, because the C terminal would not be free to access the nickel resin or the expression level was too low for purification. In fact, CrSGR protein was not detected by CBB staining on the gel (Fig. 3). Purification of the expressed CrSGR in *E. coli* remains to be achieved for further investigation of SGR

function.

4.2. Substrate specificity of SGR for correct chlorophyll metabolism

Mg of chlorophyll *a* can be easily removed under weak acidic conditions, without any enzymes (Saga and Tamiaki, 2012). Under acidic conditions, protons attack the nitrogen of pyrrole rings coordinated with Mg, and Mg is replaced with protons. The optimum pH of CrSGR was neutral, not acidic (Fig. 5A). This suggests that protons are transferred from the side chains of amino acid residues inside SGR and the protons in the solution are not directly used to remove Mg. The substrate specificity of the enzyme is important for controlling metabolic pathways. The absence of, or low CrSGR activity against chlorophyll *b* and 7-hydroxymethyl chlorophyll *a,* is essential for the proper degradation of chlorophyll *b* by repressing the production of potentially phototoxic pigments. During chlorophyll *b* degradation, chlorophyll *b* reductase reduced the formyl group at C-7 to hydroxymethyl group to produce 7-hydroxymethyl chlorophyll *a* and 7-hydroxymethyl chlorophyll *a* reductase reduced it to the methyl group to produce chlorophyll *a* (Fig. 1). Conversion of chlorophyll *b* to chlorophyll *a* is indispensable for chlorophyll *b* degradation, because pheophorbide *a* oxygenase, which is responsible for the degradation of pheophorbide *a* in the later step of chlorophyll degradation, does not catalyze molecules with the C-7 formyl group (Hortensteiner, 2006). 7-Hydroxymethyl chlorophyll *a* reductase does not reduce substrates without Mg such as 7-hydroxymethyl pheophytin *a* and 7-hydroxymethyl pheophorbide *a* (Shimoda et al., 2012). Central Mg should be coordinated to the histidine residue of this enzyme for the reaction (Wang and Liu, 2016). Meanwhile, chlorophyll *b* reductase reduces substrates without Mg (Horie et al., 2009). If SGR removes Mg from chlorophyll *b*, 7-hydroxymethyl pheophytin *a* is produced by chlorophyll *b* reductase. This pigment will not be metabolized further and would damage the cells. CrSGR slowly removed Mg from 7-hydroxymethyl chlorophyll *a*. This low activity against 7-hydroxymethyl chlorophyll *a* will not result in the accumulation of 7-hydroxymethyl pheophytin *a*, because 7-hydroxymethyl chlorophyll *a* is efficiently reduced to chlorophyll *a* and the level of 7-hydroxymethyl chlorophyll *a* is very low in the cells (Shimoda et al., 2012). Chlorophyll *a*′ was not an efficient substrate of CrSGR. However, this is not a problem as chlorophyll *a*′ is a component of the reaction center of photosystem I, and it is found at very low levels in the cell. Therefore, chlorophyll *a*′ will be degraded fully before chlorophyll *a* and *b*. After the insertion of Mg into protoporphyrin IX in the chlorophyll biosynthetic pathway, chlorophyll derivatives are the potential substrates of SGR. CrSGR did not remove Mg from the chlorophyll derivative without the phytyl side chain. Furthermore, CrSGR did not remove Mg from the chlorophyll derivative in which the D ring was oxidized (Table 1). This suggests that chlorophyll precursors in the chlorophyll biosynthetic pathway are not substrates of SGR. In this study, chlorophyll *a* is suggested to be the main substrate of CrSGR. The substrate specificity of CrSGR is considered to be related to the suppression of the formation of phototoxic molecules in chlorophyll biosynthesis and degradation processes.

4.3. Effect of substrate property to CrSGR activity

Central metal ion removal is affected by the chemical property of chlorophyll derivatives. The coordinate bond of Zn to the nitrogen of the pyrrole ring is stronger than that of Mg (Saga and Tamiaki, 2012). This is probably why Zn-chlorophyll *a* could not be a substrate of CrSGR. Under weak acidic conditions,

demetalation kinetics of chlorophyll *a*, divinyl chlorophyll *a*, and protochlorophyll *a* is reported to be chlorophyll *a* > divinyl chlorophyll *a* > protochlorophyll *a* (Saga et al., 2013). The electron-withdrawing ability of the vinyl group and the electronic states and/or flexibility of π-macrocycles are considered to affect the demetalation kinetics. Protochlorophyllide *a* and chlorophyll c_1, in which C-8 vinyl group of chlorophyll c_2 is substituted with an ethyl group, are also compared with chlorophyllide *a* in demetalation (Sadaoka et al., 2013). The kinetics is chlorophyllide *a* > protochlorophyllide *a* > chlorophyll c_1. CrSGR favored chlorophyll *a* > divinyl chlorophyll *a* > protochlorophyll *a* or chlorophyll c_2 (Table 1). This is consistent with demetalation kinetics under weak acidic conditions. These observations suggest that CrSGR removes Mg by protonation of the chlorin ring. Therefore, substrate specificity of CrSGR is related to both the fitness of the substrate with the substrate pocket and coordinate bond strength of the metal ion to the chlorin ring.

4.4. Conservation of SGR function between green algae and land plants

Chlorophyll degradation is observed in unicellular algae, such as green algae, *Chlamydomonas* (Siaut et al., 2011), diatoms, *Phaeodactylum,* and *Chaetoceros* (Nagao et al., 2010; Alipanah et al., 2015). However, the process of chlorophyll degradation in algae has not been studied in detail. In this study, CrSGR was found to be functional in *Arabidopsis* when it was transiently expressed (Fig. 8). This suggests that SGR function is conserved between green algae and land plants. CrSGR does not remove Mg from chlorophyllide *a*. This substrate specificity indicates that CrSGR is similar to the SGR subfamily, AtSGR1 and AtSGR2, but not to the SGRL subfamily, AtSGRL (Shimoda et al., 2016). These results suggest that primary SGR removed Mg from chlorophyll *a* alone, and SGRL developed later to degrade chlorophyllide *a*. Further physiological and biochemical study of SGR will provide insight into chlorophyll metabolism.

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Figure legends

Figure 1. Chlorophyll metabolic pathway

A part of the chlorophyll biosynthesis and degradation pathway is shown. Mg-dechelatase catalyzes the conversion of chlorophyll *a* to pheophytin *a*. 1, chlorophyll synthase, 2, chlorophyllide *a* oxygenase, 3, chlorophyll *b* reductase, 4, 7-hydroxymethyl chlorophyll *a* reductase, 5, pheophytinase, and 6, pheophorbide *a* oxygenase.

Figure 2. Amino acid alignment and phylogenic tree of SGR

A. Amino acid alignment of SGR. SGR homologous sequences from two green algae and four land plants were aligned. Conserved residues are closed with line. MpSGR, *Micromonas pusilla* CCMP1545 SGR

(XP_003060977); AtSGR1, *Arabidopsis thaliana* SGR1 (AT4G22920); AtSGR2, *A. thaliana* SGR2 (AT4G11910); AtSGRL, *A. thaliana* SGRL (AT4G04900); SlSGR1, *Solanum lycopersicum* iTAG2.3 (Solyc08g080090); SlSGR2, *S. lycopersicum* iTAG2.3 (Solyc12g056480); SlSGRL, *S. lycopersicum* iTAG2.3 (Solyc04g063240); OsSGR, *Oryza sativa* SGR (Os09g0532000); OsSGRL, *O. sativa* SGRL (Os04g0692600); SbSGR, *Sorghum bicolor* (Sobic.002G274800); and SbSGL, *S. bicolor* (Sobic.006G127300).
B. Phylogenic tree of SGR. A neighbor-joining tree was constructed based on the alignment shown in A. Bootstrap values for each clade are indicated on each node, and the scale bar indicates the number of amino acid substitutions per site.

Figure 3. Expression of recombinant CrSGR
CrSGR with FLAG-tag was expressed in *E. coli* and analyzed using gel electrophoresis followed by CBB staining (upper panel) or immunoblotting (lower panel). One µL of the cell lysate and 5 µL of the soluble fraction were applied for CBB staining and 0.5 µL of the cell lysate and 2.5 µL of the soluble fraction were applied for immunoblotting. One µL of the cell lysate and the soluble fraction are equivalent to 20 µL of the *E. coli* culture. CrSGR with FLAG-tag was detected using anti-FLAG antibody. Lane 1: *E. coli* with an empty vector, lane 2: *E. coli* with a CrSGR expression vector.

Figure 4. CrSGR activity of the cell lysate and the soluble fraction against chlorophyll *a*
Chlorophyll *a* was incubated with the cell lysate or the soluble fraction of the cell expressing CrSGR for 30 min. Before and after incubation, pigments were analyzed using isocratic HPLC. Pigments were monitored by fluorescence at 680 nm with excitation at 410 nm, which is optimized to detect pheophytin *a*.

Figure 5. Effects of pH and the determination of the K_m value of CrSGR for chlorophyll *a*
A. The effect of pH on CrSGR activity. The optimal pH was measured using 6 µM chlorophyll *a* under sodium phosphate or Tris-HCl buffer (n=3 ± SD).
B. Determination of K_m value of CrSGR for chlorophyll *a*. The pheophytin *a* production was measured at various chlorophyll *a* concentrations after incubation for 30 min. Three independent results are plotted using a square, triangle, and circle. The inset shows the Lineweaver-Burk plot. The line is shown using the calculated value.

Figure 6. Molecular structures of chlorophyll derivatives
Chlorophyll *a* and its derivatives, chlorophyll c_2 and pheophytin *a* are shown.

Figure 7. CrSGR activity against various chlorophyll derivatives
Chlorophyll derivatives were incubated with the cell lysate of *E. coli* possessing an empty vector, or the CrSGR expressing vector for 30 min. Before and after incubation, pigments were analyzed by HPLC with monitoring fluorescence. Excitation and emission wavelengths were optimized to detect corresponding

pheophytin derivatives. Solid and gray lines represents before and after incubation respectively. Dotted line represents the standard pheophytin derivatives. The standard pheophytin derivatives were prepared by removing Mg under acidic conditions. Pigments, except for the chlorophyll *a* and *a'* mixture, were separated with reverse phase HPLC. When chlorophyll *a* and *a'* were used as the substrate, pigments were separated with normal phase HPLC. After the treatment of chlorophyll c_2 with an acidic solution to prepare standard pheophytin c_2, two major peaks were detected by HPLC. The absorbance spectrum showed that the first major peak pointed out in the graph is pheophytin c_2.

Figure 8. CrSGR expression in *Arabidopsis*
A. Leaf color change of the transformants. Inducible CrSGR with a FLAG-tag was introduced into *Arabidopsis*. CrSGR was induced by DEX application in the transformants' excised leaves, for 24 h.
B. Chlorophyll content of the transformants. Before (control) and after DEX or mock treatment for 24 h, chlorophyll was extracted from the leaves and chlorophyll content was determined (n=3 ± SD).
C. CrSGR accumulation in the transformants. Before (control) and after DEX or mock treatment for 24 h, proteins were extracted from the leaves and CrSGR was detected by immunoblotting analysis using anti-FLAG antibody. * represents non-specific band.

Table 1. Summary of CrSGR substrate specificity
++activity, +low activity, -no activity.

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